AD-A253 782

ATION PAGE

Form Approved
OMB No. 0704-0188

DATE	3. REPORT TYPE AND DATES COVERED ANNUAL 01 Dec 90 TO 30 Nov 91

4. TITLE AND SUBTITLE
POST-DOCTORAL RESEARCH AWARD

5. FUNDING NUMBERS
GR - AFOSR-89-0187
PE - 61102F
PR - 2312
TA - A5

(2)

6. AUTHOR(S)
Dr Joan Walsh Cassedy

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Society of Toxicology
1101 14th Street, NW
Suite 1100
Washington, DC 20005

8. PERFORMING ORGANIZATION REPORT NUMBER
AFOSR-TR- 92 0655

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
T. JAN CERVENY, Lt Col, USAF
AFOSR/NL
Building 410
Bolling AFB DC 20332-6448

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

DTIC
ELECTE
JUL 23 1992
S A D

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release;
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)* For the embryolethality tests, semicarbazide and isoniazid showed a slightly less-than-additive embryolethal response. Potentially different lethal modes of action may be observed for compounds that are thought to be teratogenic by the same mode of action. For the embryolethal binary mixture test of hydroxyurea and isoniazid the 3:1 mixture showed an antagonistic response, while the 1:1 and 1:3 mixtures were response additive, as expected. The antagonistic response may have been the result of poorer absorption of hydroxyurea by the severly malformed embryos, as isoniazid had a much greater concentration (in mg/L) than did hydroxyurea, even though the effective (lethal) concentration for hydroxyurea was nearly three times that for isoniazid. Short-chain carboxylic acids showed concentration additive joint actions for induction of malformation. Combinations of DNA synthesis inhibitors showed response additive to antagonistic joint actions at malformation-inducing concentrations. Since each compound inhibits a different enzyme in the process of DNA synthesis inhibition, a response additive relationship was expected. The studies have shown that joint toxic action responses for the malformation endpoint are as seen in studies with other systems using other endpoints. The ten acid mixture study clearly showed that a series of chemicals, given at very low effect concentrations, can combine to produce a significant response.

14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

92 7 [illegible] 175

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED

JOINT ACTIONS OF DEVELOPMENTAL TOXICANTS

FINAL TECHNICAL REPORT TO
SOCIETY OF TOXICOLOGY/U.S. AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

Douglas A. Dawson, Ph.D.
University of Tennessee, College of Veterinary Medicine
Knoxville, Tennessee

June, 1991

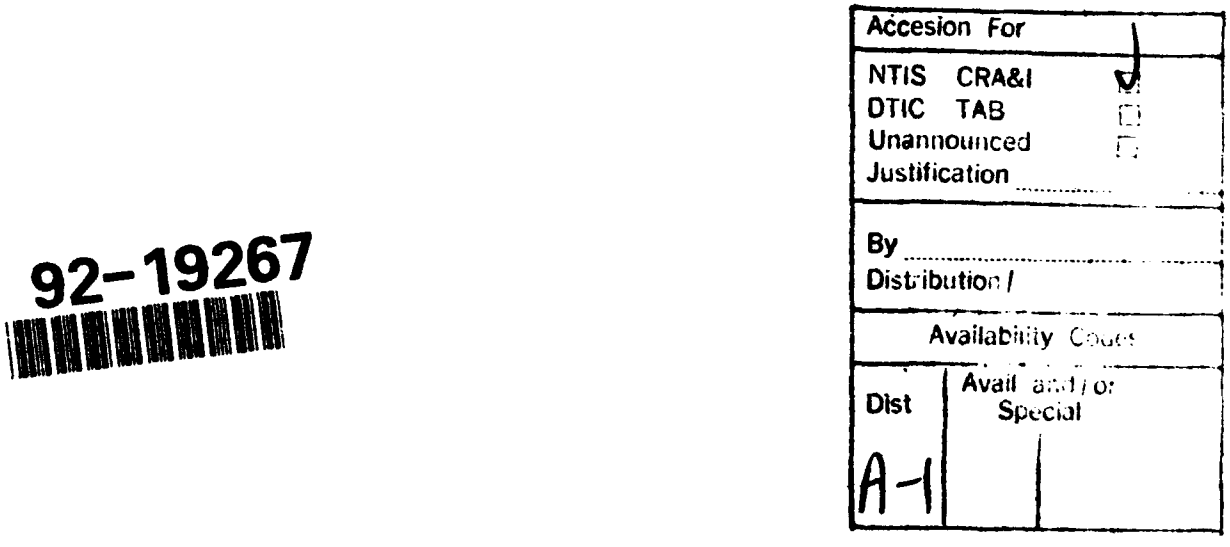

TABLE OF CONTENTS

PREFACE

This final technical report has been submitted to the Society of Toxicology and the U.S. Air Force Office of Scientific Research in fulfillment of the requirement for the Post-Doctoral Research Award presented to Douglas A. Dawson at the University of Tennessee College of Veterinary Medicine (UT/CVM).

This report is intended solely for officials of the awarding organizations as a summary of the research conducted. The author is responsible for the contents of the report, accuracy of the data generated through the award, and for proper animal care as outlined by the Animal Care and Use Committee at UT/CVM. Any comments or questions should be directed to the author.

The awardee is grateful to both organizations for this opportunity to do original research on the joint actions of developmental toxicants, using the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX). It is planned that all data presented in this report that has not already been submitted for publication, will be submitted, by the author, to peer-reviewed toxicology journals. A summary of the presentations and publications generated on this award is provided prior to the acknowledgments.

Douglas A. Dawson, Ph.D.
Research Associate

Introduction

FETAX

The Frog Embryo Teratogenesis Assay: *Xenopus*, known as FETAX [1], is a rapid, cost-effective system for evaluating the potential developmental toxicity of single chemicals, chemical mixtures, contaminated surface and groundwater, sediment leachates, and for development of structure-activity relationships. The system has applications in aquatic toxicology and experimental teratology [2-15]. A standard ASTM (American Society for Testing and Materials) guide for FETAX is nearing completion. An atlas of developmental malformations has now been published [16]. These reports will assist researchers in conducting FETAX research.

The amphibian embryo is attractive as a test organism because it has been, and remains, a classical model for experimental embryology and developmental biology. This system uses intact developing embryos, rather than cell or organ cultures, so that all developmental events, such as cleavage, blastulation, gastrulation, and organogenesis, are occuring within the 96 hour test period. These developmental events are comparable to those of other vertebrates, including mammals.

Other features that make FETAX an attractive assay are: 1) testing is rapid, with endpoints routinely obtained at 96 hours, 2) FETAX is relatively inexpensive, 3) methodology is simple, 4) large numbers of embryos (500 to 3000) are available from a single hormone-induced breeding, at any time of the year, 5) developmental stages are easily observed and scored [17], 6) pulsed exposures to a specific developmental stage are simple, as are prolonged exposures starting at specific stages, 7) FETAX allows direct exposure of the embryo, 8) embryos are compatible with low concentrations of solubility vehicles, such as DMSO, when necessary, 9) a metabolic activation system has been developed [18], 10) the embryos are functional organisms. At 96 hours, development is roughly the equivalent of the first trimester of the human, 11) finally, endpoints are easy to recognize and score.

Joint Toxic Action

Approximately 65 to 70% of human malformations are not attributable to any one known cause [19]. For this reason, the possibility that mixtures of chemical teratogens (or teratogens and co-effective agents) may be responsible for many malformations has received considerable attention. Runner and Dagg [20] hypothesized that teratogenic agents could interact in any of four ways when administered simulataneously. These joint actions were termed interference (a reduced incidence of malformations), nil effect (no increase in malformation rate over that of the most potent agent alone), additive effect (the sum of the malformation rates of the individual components) and potentiation (a more-than-additive increase in malformation rates). Incidences of each joint action type have been reported in experimental teratology [21]. Recent advances in aquatic toxicology offer a mixture testing method that can help to determine the type of joint action that may be elicited by a chemical combination. Moreover, this method provides potential insight into the mode or modes of action of the chemicals in the mixture.

The analysis of joint toxic action centers around the concepts of similarity and interaction [22-26]. For similarity, the joint action types can be similar or dissimilar, depending on whether the primary site(s) of action in the organism is(are) the same or not. As for interaction, the joint action is interactive if one toxicant influences the biological action of another. The joint action is non-interactive if there is no influence of a toxicant on the biological action of any other toxicant in the mixture. Interactive joint toxicity cannot normally be predicted by the toxicity of each component alone and is, therefore, usually only determined through testing. Models for the joint action of non-interactive toxicants, known as concentration addition and response addition, have been developed from these concepts, from the use of isobole diagrams, and from the concentration-response curves of individual toxicants [27].

Toxicants that act independently (i.e., they are non-interactive) but produce effects by the same or a very similar mode of action show strictly additive

(concentration addition) toxicity when expressed in equivalent terms and adjusted for relative potencies. For example, compounds having the non-polar narcosis mode of action show a strictly additive joint action [28-29]. This holds even for subthreshold levels of each toxicant [30]. For response addition, each toxicant in the mixture acts, primarily, upon different biological systems within the organism or affects the same system in a different way [27]. In these cases, the toxicity is less-than-additive. The differences between a concentration addition joint action and response addition can be visualized using the toxic unit model and an isobole diagram [31]. The endpoint for joint action studies need not be lethality, as sublethal measures of toxicity, such as inhibition of reproduction, immobilization, and population growth impairment, have been used in mixture studies [28,30,32].

In terms of accounting for unexplained malformations, potentiation is the joint action of most importance. Recently, teratogenic potentiation was demonstrated in rats and mice after treatment with caffeine or other methylxanthines and one of several teratogenic agents [33-34]. An interaction study conducted using FETAX produced a pattern of potentiation in *Xenopus* embryos [4] similar to that obtained in the mammalian studies. Therefore, FETAX has potential value as a model system for evaluating the teratogenic joint actions of chemical mixtures.

Research Objectives

The objectives of this research were: 1) to generate quantitative experimental data concerning the embryolethality (LC_{50}) and teratogenicity (EC_{50}) of selected binary mixtures of several developmental toxicants; 2) to determine the biological joint action (e.g., potentiation, concentration addition, response addition, or antagonism) of each mixture using toxic unit analysis, isobole diagrams, and Mixture Toxicity Index analysis; 3) explore the value of the Mortality/Malformation Index (MMI) in assessing joint action; and 4) to determine if there is any relationship between the mode of action for

teratogenicity and the type of joint action produced; as has been observed in aquatic toxicology studies.

Several developmental toxicants were selected for mixture tests based on their proposed modes of teratogenic action and on the manifestations of abnormal development each compound elicited on *Xenopus* embryos. These compounds included semicarbazide, thiosemicarbazide, isoniazid, β-aminopropionitrile, and penicillamine, all osteolathyrogenic compounds; hydroxyurea, 5-fluorouracil, and cytosine arabinoside, DNA synthesis inhibitors; 6-aminonicotinamide, a metabolic inhibitor; emetine, a protein synthesis inhibitor; all-*trans* retinoic acid, a cell membrane toxicant; nicotine, a cholinergic agonist, and valproic, 2-ethylhexanoic, butyric, and pentanoic acids, all short-chain carboxylic acids (SCCAs). Twenty binary mixtures were selected from these chemicals, to be tested at both malformation-inducing and embryolethal concentrations. However, work conducted the first year indicated that the malformation endpoint was a more suitable endpoint than embryolethality, so malformation was used as the endpoint for all remaining combinations. This allowed twenty-five binary mixtures to be tested, instead of the twenty originally proposed, and, in addition, one ten chemical mixture was tested. These additional tests were conducted to more fully evaluate the system and the nature of joint toxic action at malformation-inducing concentrations.

Future Funding Potential

There are several aspects to this research that appeared to be relevant for future funding. One was the possibility of coupling mixture teratogenesis with quantitative structure-activity relationship (QSAR) development at malformation-inducing concentrations. It is generally accepted that a simple QSAR models a specific endpoint, such as induction of malformation or lethality, for a single mode of action. Therefore, it should be possible to develop a QSAR for the osteolathyrogens that induce malformations in a manner like that for semicarbazide and, likewise, develop a QSAR for induction of malformations for carboxylic acids which act like valproic acid. In

such a case, any combination of osteolathyrogens which act like semicarbazide should have a concentration additive rate of malformation, as should any combination of carboxylic acids. However, any combination of an osteolathyrogen with a carboxylic acid would be expected to produce a less-than-additive response. Therefore, results of tests for any binary mixture with a representative compound from each QSAR should serve as a hazard assessment model for any potential combination of chemicals from those two QSAR equations. This possibility was explored further in a proposal submitted to the National Institutes of Health in June, 1990. It has now been funded for two years, ensuring that a more complete analysis of the ideas and approach, first put forth in this SOT/USAFOSR award, will be conducted. This work includes a complete analysis of joint toxic action by type or types of malformation, in order to detect any possible subcategories of joint action toxicity. It is extremely unlikely that such a proposal for FETAX research would have been funded by NIH, without the data generated from this award.

In addition, other methods of analyzing the mixture data [35-38] will be explored to discern if any other relationships can be detected. Such analysis of the data should be facilitated by the large number of data points obtained for mixture testing. The raw data will be made available to other researchers who request it for purposes of exploring other statistical techniques. One such analysis has been performed in collaboration with M.A. Shirazi, U.S. Environmental Protection Agency, Corvallis, OR. That work is now in press [39].

Materials and Methods

Assay Procedures

Adult pairs of *Xenopus laevis* were injected with 500-1000 I.U. of human chorionic gonadotropin to initiate amplexus and ovulation. Mid-to-late blastula stage embryos, developing normally, were selected under a dissecting microscope after removal of the jelly coat with a 2% (w/v) cysteine solution at pH 8.1.

FETAX solution [5] was used as the diluent for all tests, the breeding chamber water and for the cysteine solution. FETAX solution contains 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ per liter of deionized, distilled water.

The exposures were conducted in covered, glass Petri dishes (60 x 15 mm) containing 10 ml of solution and 25 embryos. The dishes were held in an environmental chamber at 23 $\pm$ 1°C. Control dishes had 10 ml of FETAX solution and 25 embryos. There were five control dishes for each test (125 embryos).

Preliminary tests established the 96 h EC_{50} or LC_{50} concentration-response range for each compound alone. The EC_{50} is the concentration inducing malformation in 50% of the surviving embryos, while the LC_{50} is the concentration causing 50% embryolethality.

For testing at teratogenic effects concentrations, separate stock solutions of each chemical were prepared at about twice the EC_{50} of the chemical. The solutions were adjusted to pH 7.2 to 7.6 as necessary. For each binary combination (e.g., semicarbazide and isoniazid) 3:1, 1:1, and 1:3 stock mixtures were prepared from the single compound stock solutions. Five concentrations of each mixture were tested. These concentrations were designed so that a linear range from 0 to 100% of the test embryos would become malformed. In addition, five concentrations of each individual component alone, were also tested (25 embryos per concentrations) to serve as positive controls, i.e., 1:0 and 0:1 solutions.

Mixture testing at embryolethal effects concentrations was conducted in the same manner. Individual stock solutions were prepared at 1.5 to 2 times the compound LC_{50}. The stock mixture concentrations were designed to produce a linear range from 0 to 100% embryolethality.

All exposures were 96 h in duration. At 24, 48, and 72 hours dead embryos, if any, were removed. The test solutions were changed daily using fresh preparations. At

96 h, surviving embryos were fixed in formalin and examined for gross abnormalities under a dissecting microscope. The numbers of dead and surviving malformed embryos were recorded for each dish.

Data Analysis

Three or four separate tests were conducted for each combination. For each ratio (1:0, 0:1, 3:1, 1:1, 1:3), the appropriate 96 h EC_{50} or LC_{50} was determined, using the combined data from the separate tests [7,8]. The 95% fiducial interval was also calculated by probit analysis of Statistical Analysis System (SAS) software.

Toxic unit (TU) analysis was used to determine the type of interaction for each combination [32]. In TU analysis, the EC_{50} of each compound alone is defined as 1.0 toxic unit for malformation due to exposure to that compound (the LC_{50} is 1.0 toxic unit for embryolethality). The concentration of each component at 50% effect for the mixture is given as a fraction of its EC_{50} (or LC_{50}) value (see Results section of Appendix 4 for detailed example). The sum of the component fractions gives a TU value for that ratio. Mixtures with TU values equal to 1.0 (fiducial interval overlaps 1.0) have concentration additive (CA) rates of malformation (or embryolethality). A mixture TU value <1 or >1 represents more-than-additive (potentiation) or less-than-additive toxicity, respectively. An isobole diagram can be coupled with TU analysis to determine if the less-than-additive response indicates no interaction or antagonism [29].

Results and Discussion

Control Data

Nearly 14,000 control embryos have been used in mixture tests to date. The overall control embryo malformation was <4% and control embryolethality was <1%. No tests were accepted with control embryo malformation or lethality at >10%, based on 375 or 500 control embryos per binary mixture analysis (125 per replicate test).

Summary of Test Results

The results of the 25 binary mixture tests performed to date are shown in Tables 1 and 2. The summary data tables and raw data tables can be obtained from the author, upon written request.

Embryolethality Test Results

For the embryolethality tests, semicarbazide and isoniazid showed a slightly less-than-additive embryolethal response. Although both compounds are osteolathyrogenic, osteolathyrogenicity is not necessarily the mode of action that causes embryo death. Therefore, potentially different lethal modes of action may be observed for compounds that are thought to be teratogenic by the same mode of action. In fact, at near embryolethal concentrations for isoniazid, the gross appearance of the severly malformed embryos is more typical of that for a weak carboxylic acid, such as valproic acid. Since isoniazid is short for isonicotinic acid hydrazide, it has a carbonyl (C=O) group. Therefore, the mode of action for embryolethality of isoniazid may be different from that for semicarbazide.

For the embryolethal binary mixture test of hydroxyurea (a DNA synthesis inhibitor) and isoniazid (an osteolathyrogen) the 3:1 mixture (hydroxurea:isoniazid) showed an antagonistic response, while the 1:1 and 1:3 mixtures were response additive, as expected. The antagonistic response may have been the result of poorer absorption of hydroxyurea by the severly malformed embryos, as isoniazid had a much greater concentration (in mg/L) than did hydroxyurea, even though the effective (lethal) concentration for hydroxyurea was nearly three times that for isoniazid.

The use of embryos for these tests is no longered deemed appropriate for lethality tests. Death may result from an acute mode of action or from poor development, or from a combination of the two. If the latter is the case, upto four modes of action may be involved (two for each compound - one for malformation-induced death and one for an acute lethal mode) in the observed response

(embryolethality). Therefore, it is not possible to relate joint embryolethality with modes of action. It is suggested, however, that use of seven day old *Xenopus* tadpoles might be appropriate for analyzing the joint acute lethal actions of toxicants [40]. Seven day old tadpoles have completed most of organogenesis and, therefore, are usually not susceptible to malformation. An exception is limb development that takes place at metamorphosis. These findings have resulted in a change in emphasis of this research (exclusively to examination of mixture toxicity for the malformation endpoint) for the remainder of the study. This change provided more appropriate information than continued embryolethal testing would have, as originally proposed as an aspect of this project.

Malformation Test Results

The osteolathyrogen semicarbazide was tested with three other osteolathyrogens: isoniazid, β-aminopropionitrile and penicillamine. Concentration additive (strictly additive) rates of malformation were observed for semicarbazide with isoniazid and semicarbazide with β-aminopropionitrile. In contrast, semicarbazide and penicillamine showed a response additive (less-than-additive) rate of malformation, as did β-aminopropionitrile with penicillamine. This is thought to be due to a difference in the specific mode (mechanism) of action for the compounds (see submitted paper for details). There are at least three or four possible specific modes of action for osteolathyrogens. The results obtained in this study show that this mixture testing procedure is able, at least in this instance, to discriminate between these mechanisms. Therefore, the procedure may be of significant value in delineating agents with common mechanisms of teratogenesis.

Thiosemicarbazide, an osteolathyrogen was also tested with β-aminopropionitrile and isoniazid, and isoniazid was tested with β-aminopropionitrile. Concentration additive joint actions were observed, indicating that these compounds induce osteolathyrism in a similar manner.

The short-chain carboxylic acids (SCCA), valproic acid, 2-ethylhexanoic acid, pentanoic acid and butyric acid, also showed concentration additive joint actions for induction of malformation. Although the exact mechanism for SCCA teratogenesis is not known [but is thought to be related to folate metabolism or synthesis (H. Nau, personal communication)], it would not be surprising that these structurally similar compounds have the same mode of action for induction of malformation. This idea formed the basis for the ten chemical mixture study (see below).

The combinations of DNA synthesis inhibitors (hydroxyurea, cytosine arabinoside, 5-fluorouracil) showed response additive to antagonistic joint actions at malformation-inducing concentrations. Since each compound inhibits a different enzyme in the process of DNA synthesis inhibition, a response additive relationship was expected. The antagonistic response, which indicates the compounds are interactive, is of potential importance. Comparison of this malformation data with cancer chemotherapeutic efficacy for combinations of DNA synthesis inhibitors is needed to determine if there is any relationship of teratogenic potential with therapeutic value.

Five binary mixture tests containing all-*trans* retinoic acid have been conducted. Retinoic acid and nicotine showed no interaction (i.e., there was no effect greater than that seen for the most potent agent in a mixture). Since nicotine is a cholinergic agonist and retinoic acid inhibits chondrogenesis and is a cell membrane toxicant, a less-than-additive effect was expected and observed. The important result for this combination is that the primary malformation elicited by these two compounds on *Xenopus* embryos was the same. Both compounds affect development of the mouth, although other malformations have been observed at higher concentrations. This indicates that, even though the primary site of action was the same, the effect is not elicited in the same manner. For a less-than-additive response, the primary effect is either on different systems or on the same system through a different mode of action [27].

Table 1. Joint Action Response for 25 Binary Mixture Tests.

Binary Mixture	Joint Action
Embryolethality Tests	
Semicarbazide:Isoniazid	RA
Hydroxyurea:Isoniazid	RA - ANT
Malformation Tests	
Semicarbazide:Isoniazid	CA
Semicarbazide:β-Aminopropionitrile	CA
Isoniazid:β-Aminopropionitrile	CA
Isoniazid:Thiosemicarbazide	CA
Thiosemicarbazide:β-Aminopropionitrile	CA
Semicarbazide:Penicillamine	RA
β-Aminopropionitrile:Penicillamine	RA
Valproic Acid:Butyric Acid	CA
Valproic Acid:2-Ethylhexanoic Acid	CA
Valproic Acid:Pentanoic Acid	CA
Butyric Acid:Pentanoic Acid	CA
Hydroxyurea:Cytosine Arabinoside	RA - ANT
Hydroxyurea:5-Fluorouracil	RA - ANT
Cytosine Arabinoside:5-Fluorouracil	ANT
Retinoic Acid:Nicotine	NI
Retinoic Acid:Isoniazid	RA
Retinoic Acid:Hydroxyurea	RA
Retinoic Acid:6-Aminonicotinamide	RA
Retinoic Acid:Valproic Acid	RA
Isoniazid:Valproic Acid	RA
Isoniazid:Hydroxyurea	RA
Isoniazid:6-Aminonicotinamide	RA
Hydroxyurea:Valproic Acid	RA

CA - Concentration Addition
RA - Response Addition
NI - No Interaction
ANT - Antagonism

Table 2. Comparison of Toxic Unit Values for Binary Mixtures with Types of Joint Actions at Malformation-Inducing Concentrations.

Joint Action Type	Toxic Unit Values		
Binary Mixture	3:1	1:1	1:3
Concentration Addition			
Semicarbazide:Isoniazid	1.08	1.03	1.02
Semicarbazide:β-Aminopropionitrile	1.01	0.99	0.96
Thiosemicarbazide:Isoniazid	1.01	1.02	0.96
Thiosemicarbazide:β-Aminopropionitrile	0.99	0.96	1.06
Isoniazid:β-Aminopropionitrile	0.97	1.01	1.00
Valproic Acid:Butyric Acid	1.01	0.96	0.98
Valproic Acid:2-Ethylhexanoic Acid	0.98	1.02	1.01
Valproic Acid:Pentanoic Acid	1.02	1.03	0.98
Butyric Acid:Pentanoic Acid	0.98	1.06	1.06
Response Addition			
Semicarbazide:Penicillamine	1.23	1.35	1.23
β-Aminopropionitrile:Penicillamine	1.27	1.32	1.22
Retinoic Acid:Isoniazid	1.23	1.22	1.30
Retinoic Acid:Hydroxyurea	1.25	1.39	1.35
Retinoic Acid:6-Aminonicotinamide	1.23	1.36	1.26
Retinoic Acid:Valproic Acid	1.29	1.32	1.32
Isoniazid:Hydroxyurea	1.15	1.29	1.29
Isoniazid:6-Aminonicotinamide	1.23	1.26	1.14
Isoniazid:Valproic Acid	1.33	1.53	1.19
Hydroxyurea:Valproic Acid	1.29	1.28	1.14
No Interaction			
Retinoic Acid:Nicotine	1.35	1.76	1.37
Response Addition - Antagonism			
Hydroxyurea:Cytosine Arabinoside	1.59	2.07	1.32
Hydroxyurea:5-Fluorouracil	1.37	1.65	1.61
Antagonism			
Cytosine Arabinoside:5-Fluorouracil	1.72	2.04	1.58

For the other four binary mixtures containing retinoic acid, response additive rates of malformation were observed. These results are consistent with the hypothesis, since isoniazid is an osteolathyrogen, hydroxyurea a DNA synthesis inhibitor, 6-

aminonicotinamide an antimetabolite and valproic acid, a possible inhibitor of folate metabolism. Retinoic acid induced malformations have not been shown to be brought about by any of these modes of action.

Isoniazid was also tested with three compounds which are not osteolathyrogens, hydroxyurea, valproic acid, and 6-aminonicotinamide. In each case, response additive rates of malformation were observed. These results are also consistent with the hypothesis.

The DNA synthesis inhibitor hydroxyurea also showed a response additive joint action with valproic acid, as expected. This indicates that valproic acid induced teratogenesis is probably not associated with the inhibition of DNA synthesis, a result in agreement with mammalian studies [41].

With the exception of the DNA synthesis inhibitors, in which antagonistic joint actions were observed, all results were as expected according to the hypothesis. The antagonistic joint actions represent the phenomenon known as interaction. Such results are not predictable without testing. Overall, there appears to be a relationship between common and different modes of teratogenesis and the types of joint actions produced by binary mixtures of teratogens.

Ten Acid Mixture Study

The results of the binary mixtures studies indicated that the four short-chain carboxylic acids tested together all showed concentration additive joint actions. For this reason, the malformation endpoint for a series of ten such acids were examined in a mixture. Joint action theory predicts that such a series should show a concentration additive joint action, if they all act similarly. Preliminary testing showed a common dysmorphology induced by these acids in *Xenopus* embryos (as had also been seen in mammalian embryo-culture [41]), adding to the likelihood these acids all acted similarly. The test (see paper) showed concentration addition, as expected.

Implications of Results

The studies have shown that joint toxic action responses for the malformation endpoint are as seen in studies with other systems using other endpoints. Calabrese [42] has recently reviewed mixture toxicity data from a variety of toxicology subdisciplines, and stressed the need for effective communication of the results between disciplines. The results of this study were in agreement with those of previous studies. The data is important because, in the field of teratology, no such demonstration of this system of joint actions had been previously deduced. In fact, mammalian teratology screens, as presently used, were not directed toward such an analysis. The FETAX system was ideally suited for such studies because no malformations are induced as the result of maternal toxicity, no maternal metabolism is incorporated, which would confound identification of the relationship, and no problems with toxicant binding to components of the mammalian embryo-culture system are present to alter the response. The individual papers (listed below) include other points about the significance of these results.

The ten acid mixture study clearly showed that a series of chemicals, given at very low effect concentrations, can combine to produce a significant response. In fact, this response was equivalent to that of any one such acid given alone. This may have potential significance in the study of mixture teratology and for the design of future epidemiological studies.

Presentations, Publications, and Grants

The following presentations, describing research results generated under this award, have been made at scientific meetings:

1). Dawson, D.A. and T.W. Schultz. Teratogenic interactions and mechanisms of action: A potential relationship. In Vitro Teratology Conference, Research Triangle Park, NC., Sept. 21, 1989.

2). Dawson, D.A. and T.W. Schultz. Mixture teratogenesis testing with FETAX: The relationship of joint actions of teratogens to mechanisms of action. Soc. Environ. Toxicol. Chem., Toronto, Canada, Oct. 31, 1989.

3). Dawson, D.A., T.W. Schultz, and T.S. Wilke. Teratogenic interactions of DNA synthesis inhibitors. Soc. Toxicol., Miami Beach, FL., Feb. 14, 1990.

4). Dawson, D.A., T.S. Wilke, and T.W. Schultz. Developmental malformation as an endpoint in aquatic toxicology: Method development for mixture teratogenesis. Soc. Environ. Toxicol. Chem., Arlington, VA., Nov. 14, 1990.

5). Dawson, D.A., T.S. Wilke, and T.W. Schultz. Joint action of toxicants in *Xenopus* embryos. Soc. Environ. Toxicol. Chem., Arlington, VA., Nov. 15, 1990.

6). Dawson, D.A. and T.S. Wilke. Joint actions of developmental toxicants: Evaluation of binary mixtures. Soc. Toxicol., Dallas, TX., Feb. 28, 1991.

7). Dawson, D.A. Additive malformation rate for a mixture of ten aliphatic acids. Teratology Soc., Boca Raton, FL., June 27, 1991.

The following papers are a result of the work conducted on this award:

1). Dawson, D.A. and T.S. Wilke. Initial evaluation of developmental malformation as an endpoint in mixture toxicity hazard assessment for aquatic vertebrates. *Ecotox. Environ. Saf.*, 21:215:226 (1991).

2). Dawson, D.A. and T.S. Wilke. Evaluation of FETAX (Frog Embryo Teratogenesis Assay: *Xenopus*) as a model system for mixture toxicity hazard assessment. *Environ. Toxicol. Chem.*, 10:in press (1991).

3). Dawson, D.A. and T.S. Wilke. Joint toxic action of binary mixtures of osteolathyrogens at malformation-inducing concentrations for *Xenopus* embryos. *J. Appl. Toxicol.*, in press.

4). Dawson, D.A. Additive incidence of developmental malformation for *Xenopus* embryos exposed to a mixture of ten aliphatic carboxylic acids. *Teratology*, in press.

5). Dawson, D.A. and T.S. Wilke. Joint actions of developmental toxicants in *Xenopus* embryos: Binary mixtures of DNA synthesis inhibitors. submitted.

6). Dawson, D.A. and T.S. Wilke. Evaluation of mixtures of valproic acid and all-*trans* retinoic acid on *Xenopus* embryo development. In preparation.

7). Dawson, D.A. and T.S. Wilke. Evaluation of mixtures of hydroxyurea and valproic acid on *Xenopus* embryo development. In preparation.

In addition, a paper has been prepared as a mathematical model for mixture toxicity, that used data generated on this award to evaluate the model. It is identified below.

8). Shirazi, M.A. and D.A. Dawson. Developmental malformation of frog embryos: An analysis of teratogenicity of chemical mixtures. *Arch. Environ. Contam. Toxicol.*, in press.

The following grant proposal has been funded by the National Institute of Environmental Health Sciences, as a result of preliminary data generated on this award.

Dawson, D.A. (PI). Mixture Teratogenesis: Relation to Mechanisms and QSARs. 4/91-3/93, $138,000.

Acknowledgments

The author would like to acknowledge the technical assistance of Teresa Wilke and the word-processing assistance of Wanda Aycock.

References

1. Dumont JN, Schultz TW, Buchanan MV, Kao GL: Frog embryo teratogenesis assay: *Xenopus* (FETAX) - A short-term assay applicable to complex environmental mixtures. In Waters MD, Sandhu SS, Lewtas J, Claxton L, Chernoff N and Nesnow S: "Short-term Bioassays in the Analysis of Complex Environmental Mixtures III." New York: Plenum Publishing, 1983, pp 393-405.

2. Courchesne CL, Bantle JA: Analysis of the activity of DNA, RNA and protein synthesis inhibitors on *Xenopus* embryo development. Teratogenesis Carcinog Mutagen 5:177-193, 1985.

3. Dawson DA, Fort DJ, Smith GL, Newell DL, Bantle JA: Evaluation of the developmental toxicity of nicotine and cotinine with frog embryo teratogenesis assay: *Xenopus*. Teratogenesis Carcinog Mutagen 8:329-338, 1988.

4. Dawson DA, Bantle JA: Coadministration of methylxanthines and inhibitor compounds potentiates teratogenicity in *Xenopus* embryos. Teratology 35:221-227, 1987.

5. Dawson DA, Bantle JA: Development of a reconstituted water medium and preliminary validation of the frog embryo teratogenesis assay: *Xenopus* (FETAX). J Appl Toxicol 7:237-244, 1987.

6. Sabourin TD, Faulk RT: Comparative evaluation of a short-term test for developmental effects using frog embryos. In McLachlin JM, Pratt RM, Markett CL: "Banbury Report 26: Developmental Toxicology: Mechanisms and Risk." New York: Cold Spring Harbor Laboratory, 1987, pp 203-223.

7. Schultz TW, Dumont JN, Epler RG: The embryotoxic and osteolathyrogenic effects of semicarbazide. Toxicology 36:183-198, 1985.

THIS PAGE IS MISSING IN ORIGINAL DOCUMENT

pg. 21

19. Brent RL: The application of basic developmental biology data to clinical teratological problems. In Welsch F: "Approaches to Elucidate Mechanisms in Teratogenesis." Washington, D.C., Hemisphere, 1987, pp 255-267.

20. Runner MN, Dagg CP: Metabolic mechanisms of teratogenic agents during morphogenesis. In Kaliss N: "Normal and Abnormal Differentiation and Development." National Cancer Institute Monograph No. 2, Washington, D.C., U.S. Gov't Printing Office, 1960, pp 41-54.

21. Wilson JG: Teratogenic interactions of chemical agents in the rat. J Pharmacol exp Ther 144:429-436, 1964.

22. Bliss CI: The toxicity of poisons applied jointly. Ann Appl Biol 26:585-615, 1939.

23. Plackett RL, Hewlett PS: Statistical aspects of the independent joint action of poisons, particularly insecticides. I. The toxicity of a mixture of poisons. Ann Appl Biol 35:347-358, 1948.

24. Plackett RL, Hewlett PS: Quantal response to mixtures of poisons. J Roy Statist Soc, Series B 14:141-154, 1952.

25. Plackett RL, Hewlett PS: A comparison of two approaches to the construction of models for quantal responses to mixtures of drugs. Biometrics 23:27-44, 1967.

26. Hewlett PS, Plackett RL: A unified theory for quantal responses to mixtures of drugs: competitive action. Biometrics 20:566-575, 1964.

27. Anderson PD, Weber LJ: The toxicity to aquatic populations of mixtures containing certain heavy metals. Proc Int Conf on Heavy Metals in the Environ 2:933-953, 1975.

28. Hermens J, Canton H, Stegger N, Wegman R: Joint effects of a mixture of 14 chemicals on mortality and inhibition of reproduction of *Daphnia magna*. Aquat Toxicol 5:315-322, 1984.

29. Broderius S, Kahl M: Acute toxicity of organic chemical mixtures to the fathead minnow. Aquat Toxicol 6:307-322, 1985.

30. Deneer JW, Sinnige TL, Seinen W, Hermens JLM: The joint acute toxicity to *Daphnia magna* of industrial organic chemicals at low concentrations. Aquat Toxicol 12:33-38, 1988.

31. Sprague JB: Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. Water Res 4:3-32, 1970.

32. Schultz TW, Allison TC: Toxicity and toxic interaction of aniline and pyridine. Bull Environ Contam Toxicol 23:814-819, 1979.

33. Ritter EJ, Scott WJ, Wilson JG, Mathinos PR, Randall JL: Potentiative interactions between caffeine and various teratogenic agents. Teratology 25:229-238, 1982.

34. Nakatsuka T, Handa S, Fujii T: Potentiating effects of methylxanthines on teratogenicity of mitomycin C in mice. Teratology 28:243-247, 1983.

35. Bernebaum MC: Isobolographic, algebraic, and search methods in the analysis of multiagent synergy. J Am Coll Toxicol 7:927-938, 1988.

36. Carter WH Jr, Gennings C, Staniswalis JG, Campbell ED, White KL Jr: A statistical approach to the construction and analysis of isobolograms. J Am Coll Toxicol 7:963-973, 1988.

37. Gessner PK: A straightforward method for the study of drug interactions: An isobolographic analysis primer. J Am Coll Toxicol 7:987-1012, 1988.

38. Shirazi MA, Linder G: An analysis of biological response to chemical mixtures. Toxicologist 10:83, 1990.

39. Shirazi MA, Dawson DA: Developmental malformation of frog embryos: An analysis of teratogenicity of chemical mixtures. Arch Environ Contam Toxicol in press.

40. Dawson DA, Schultz TW, Baker LL, Wilke TS: Comparative developmental toxicity of acetylenic alcohols on embryos and larvae of *Xenopus laevis*. In: "Aquatic Toxicology and Hazard Assessment, 13th Volume.", 1990, (in press).

41. Brown NA, Coakley ME, Clarke DO: Structure-teratogenicity relationships of valproic acid congeners in whole-embryo culture. In Welsch F: "Approaches to Elucidate Mechanisms in Teratogenesis." Washington, D.C., Hemisphere, 1987, pp 17-31.

42. Calabrese EJ: *Multiple Chemical Interactions*. Lewis Publishers, Chelsea, MI., 704 pp.